Overcoming heparin-associated RT-qPCR inhibition and normalisation issues for microRNA quantification in patients with acute myocardial infarction

Running title: Bivalirudin or Heparinase for miRNA quantification in STEMI

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ABSTRACT

Background: Cardiac-enriched microRNAs (miRNAs) are released into the circulation following ST-elevation myocardial infarction (STEMI). Lack of standardized approaches for reverse transcription quantitative real time polymerase chain reaction (RT-qPCR) data normalisation and presence of RT-qPCR inhibitors (e.g. heparin) in patient blood samples have prevented reproducible miRNA quantification in this cohort and subsequent translation of these biomarkers to clinical practice.

Methods: Using a RT-qPCR miRNA screening platform we identified and validated an endogenous circulating miRNA as a normalisation control. In addition, we assessed the effects of *in vivo* and *in vitro* anticoagulant drugs administration (heparin and bivalirudin) on three RT-qPCR normalisation strategies [global miRNA mean, exogenous spike-in control (cel-miR-39), and endogenous miRNA control]. Finally, we evaluated the effect of heparin and its *in vitro* inhibition with heparinase on the quantification of cardiac-enriched miRNAs in STEMI patients.

Results: miR-425-5p was validated as an endogenous miRNA control. Heparin administration *in vitro* and *in vivo* inhibited all RT-qPCR normalisation strategies. In contrast, bivalirudin had no effects on cel-miR-39 or miR-425-5p quantification. *In vitro* RNA sample treatment with 0.3U of heparinase overcame heparin-induced overestimation of cardiac-enriched miRNA levels and improved their correlation with high-sensitivity troponin T.

Conclusion: MicroRNA quantification in STEMI patients receiving heparin is jeopardised by its effect on all RT-qPCR normalisation approaches. Use of samples from bivalirudin-treated patients or *in vitro* treatment of heparin-contaminated samples with heparinase are suitable alternatives for miRNA quantification in this cohort. Finally, we reinforce the evidence that cardiac-enriched miRNAs early after myocardial reperfusion reflect the severity of cardiac injury.

Keywords: microRNA; RT-qPCR normalisation; heparin; bivalirudin; myocardial infarction

1. Introduction

Coronary artery disease (CAD) is the leading cause of mortality worldwide (1). Its most serious manifestation, ST elevation myocardial infarction (STEMI), is characterized by irreversible myocardial injury due to prolonged ischaemia (1). Although cardiac troponins are established biochemical markers of STEMI, methodological limitations in their quantification (2) and only moderate correlations with infarct size (3) prompt the identification of new markers of cardiac injury.

In the last decade, microRNAs (miRNAs), small non-coding RNAs of approximately 22 – 23 nucleotides in length, have emerged as potential disease markers given their successful isolation from biological fluids and notable resistance to degradation (4-7). In the context of STEMI, many studies have reported deregulated plasmatic levels of several miRNAs (8). Amongst these, cardiac-enriched miRNAs have been shown to be up-regulated early after the onset of STEMI and are of particular interest as they are more likely to inform about the nature and extent of myocardial injury (8). Nonetheless, lack of consensus regarding real time quantitative polymerase chain reaction (RT-qPCR) data normalisation and presence of RT-qPCR inhibitors, such as heparin, in STEMI patient samples contribute to inconsistencies among studies and represent critical limitations for the translation of miRNAs to daily clinical practice (8, 9).

RT-qPCR is the current preferred method for miRNA quantification due to its accuracy, specificity, and broad dynamic range (10). Data normalisation is a fundamental step to minimize the effects of systematic errors and obtain biologically meaningful miRNA expression in RT-qPCR studies (10). The most common normalisation strategy is the use of a stably expressed endogenous control. Nonetheless, there are no circulating miRNAs that have been systematically

validated as endogenous controls in STEMI patients to date. As an alternative, synthetic miRNAs, especially *Caenorhabditis elegans* miR-39 (cel-miR-39), have been used as normalisation controls in most studies (8). However, heparin, routinely administered during coronary intervention such as primary percutaneous coronary intervention (PPCI), has been shown to affect cel-miR-39 detection by RT-qPCR, which might compromise its use as a normalisation control (11-13). Another proposed normalisation approach is to use the average expression of quantified miRNAs, based on the premise that the mean expression of hundreds to thousands of quantified miRNAs is not altered by deregulation of few miRNAs or miRNA clusters between cases and controls (14). Yet, this strategy is not feasible for clinical routine or large clinical studies focusing on few target miRNAs. Furthermore, the effect of heparin on the global miRNA expression is also not known. Therefore, circulating miRNA quantification in STEMI patients undergoing PPCI remains an unmet challenge. This study aimed to (i) identify and validate an endogenous circulating miRNA control in STEMI patients; (ii) assess the effects of anticoagulant drugs, such as heparin and bivalirudin, on currently used RT-qPCR normalisation approaches; and (iii) evaluate the effect of *in vitro* heparin inhibition with heparinase on the quantification of circulating cardiac-enriched miRNAs in STEMI patients.

2. Materials and Methods

2.1. Study cohort and design

Patient recruitment was performed at the Freeman Hospital, Newcastle upon Tyne, United Kingdom. Only patients presenting within 6 hours of chest pain onset which met electrocardiographic criteria of STEMI, as well as angiographic evidence of a large (≥ 3 mm calibre) and acutely occluded (TIMI flow 0/1) coronary artery undergoing PPCI were included. This study was approved by the local ethics

committee and verbal consent was obtained once patients met inclusion criteria, followed by written consent post procedure (REC reference: NE/14/1070; EudraCT number: 2014-002628-29). Plasma samples were collected from 73 STEMI patients prior to and at multiple time points following myocardial reperfusion. Patients received an intravenous *bolus* of unfractionated heparin (70 units/kg, n = 70) or bivalirudin (0.75 mg/kg, n = 3) prior to PPCI, which preceded collection of the first blood sample. In addition, plasma samples from healthy individuals (n = 6) and patients with stable CAD (n = 6) were used as controls.

The study was conducted in 3 consecutive phases (**Figure 1**). During phase 1, screening of 179 miRNAs using RT-qPCR panels was performed in STEMI patients across 6 time points prior to and post-PPCI as well as in stable CAD controls to identify candidate endogenous miRNA controls (**Figure 1A**). The 4 most stable miRNAs identified in the screening phase were subsequently quantified in 34 STEMI patients to assess miRNA stability and validate a suitable miRNA endogenous control (validation phase – **Figure 1B**). The effects of heparin and bivalirudin administration on the three normalisation approaches (global miRNA mean, cel-miR-39, and validated endogenous control) were also analysed as part of the validation phase (**Figure 1B**). Finally, the effect of the *in vitro* treatment of heparin-contaminated samples with heparinase on cardiac-enriched miRNA (miR-1 and miR-133b) quantification and its impact on the correlation between these miRNAs and high-sensitivity troponin T were evaluated in 70 STEMI patients (application phase - **Figure 1C**).

2.2. Plasma sample preparation and RNA isolation

Blood samples were collected in EDTA-coated tubes (Becton Dickinson, USA) from a central arterial source up to 90 minutes post-PPCI using a 6F radial artery sheath

left in from the PPCI procedure for this purpose. The 120min, 180min, and 24h post-PPCI samples from STEMI patients were collected from a peripheral venous source (antecubital vein). Immediately after collection, whole blood was centrifuged at 1,500xg for 15 minutes at room temperature for plasma separation. Plasma samples were aliquoted and stored at -80°C until analysis.

Following quick thaw of plasma samples at 37°C, total RNA was isolated from 200 µL of plasma using the miRNeasy serum/plasma kit (Qiagen, Germany), according to the manufacturer's protocol. Prior to addition of chloroform and aqueous phase separation, 3.5µL (1.6×10^8 copies/µL) of synthetic cel-miR-39 (Qiagen, Germany) were added to all samples. Assessment of total RNA concentration and integrity was performed by a 2100 Bioanalyzer instrument using the RNA 6000 Pico Kit (Agilent technologies, Germany) (**Supplemental table 1**). Additional details of the RNA extraction and concentration assessment procedures are included in the **Supplemental methods**.

2.3. miRNA quantification

For miRNA screening, 4µL of total RNA were reverse transcribed using the Universal cDNA synthesis kit (Exiqon, Denmark). A total of 179 circulating miRNAs were quantified by RT-qPCR using serum/plasma focused miRNA PCR panels (Exiqon, Denmark) and the Exilent SYBR Green master mix (Exiqon, Denmark). To control for variations in efficiency at each experimental level, miRCURY LNA™ RNA spike-ins (Exiqon, Denmark) were equally added to the samples prior to RNA extraction (cel-miR-39), cDNA synthesis (UniSp6), and PCR (UniSp3). Data quality control assessment was based on the quantification cycle (Cq) values obtained for these RNA templates across all samples (**Supplemental figure 1**). In addition, to minimize PCR inter-run variability, inter-plate calibration was performed according to the Cq

values obtained for UniSp3. The global miRNA expression mean was calculated as the geometric mean of Cq values obtained for all miRNAs, excluding miRNAs with Cq > 35.

In the validation and application phases, 5µL of total RNA were reverse transcribed using the TaqMan® microRNA reverse transcription kit (Applied Biosystems, USA) and stem-loop specific TaqMan small RNA primers (Applied Biosystems, USA). Each miRNA was quantified using specific TaqMan microRNA assays (Applied Biosystems, USA) and the SensiFAST probe Hi-ROX master mix (Bioline, United Kingdom) in triplicates by a 7500 real-time PCR system (Applied Biosystems, USA). Calibration curves and efficiency of each assay and intra-assay variability are displayed in **Supplemental figure 2** and **Supplemental table 2**, respectively.

2.4. miRNA stability assessment

To identify and validate the most stable endogenous miRNA, variability and stability assessments of candidate miRNAs were performed by calculation of the coefficient of variability (CV) and by the NormFinder v20 (15), geNorm v3.5 (16), and BestKeeper (17) software, respectively. These software applications utilize solid statistical algorithms to determine the variation in expression of multiple candidate normalisation genes across different samples. Lower stability scores in NormFinder (*S* score), geNorm (*M* score), and BestKeeper [standard deviation - std dev ($\pm$ CP)] are expected for more stable miRNAs.

2.5. Heparin ELISA

A solid-phase heparin enzyme-linked immunosorbent assay (ELISA) kit (BlueGene, China) containing wells pre-coated with monoclonal anti-porcine heparin antibody was used to confirm the presence and determine the concentration of heparin in both plasma (100µL) and RNA (10µL) samples from STEMI patients (n = 10).

Furthermore, the effect of RNA treatment with serial doses of heparinase I (Sigma-Aldrich, USA) on heparin concentration in these samples was also assessed by ELISA. Different doses of heparinase I (0U, 0.25U, 0.5U, and 1U) were added to 10 μ L of heparin-contaminated samples from the same STEMI patients (n = 3), incubated for 1 hour at room temperature. Heparin concentration was subsequently determined by ELISA. Detailed descriptions of the protocols for RNA treatment with heparinase and heparin ELISA are included in the **Supplemental methods**.

2.6. Effect of *in vitro* administration of heparin and bivalirudin on miRNA expression

To assess the effect of *in vitro* heparin administration on miRNA detection by RT-qPCR, serial doses of unfractionated heparin (Sigma-Aldrich, USA) were added to heparin-free RNA samples from STEMI patients (n = 3) and the difference in Cq values (Δ Cq) to the heparin-free samples from the same STEMI patients were calculated after RT-qPCR. In addition, serial doses of heparinase I were added to heparin-contaminated RNA samples from STEMI patients (n = 3) to test whether and at which dose heparinase could reverse any potential effect of heparin on miRNA detection. Finally, to assess whether *in vitro* addition of bivalirudin (0.1 and 1 μ g/ μ L - Sigma-Aldrich, USA) to RNA samples would have any effect on cel-miR-39 or the endogenous miRNA control expression, the Δ Cq between bivalirudin-free and bivalirudin-treated RNA samples from the same patients with stable CAD (n = 3) were determined. Detailed information regarding the *in vitro* experiments with heparin and bivalirudin can be found in the **Supplemental methods**.

2.7. Effect of *in vivo* administration of heparin and bivalirudin on miRNA expression

After identification of an adequate dose of heparinase that could rescue miRNA detection by RT-qPCR in heparin-contaminated samples (0.3U), the effect of *in vivo* heparin administration on miRNA expression was analysed by comparing the ΔCq between heparin-contaminated and heparinase-treated RNA samples from the same STEMI patients (n = 70). To evaluate the effect of *in vivo* bivalirudin administration on the newly identified endogenous miRNA control, samples were obtained at 13 successive time points from STEMI patients receiving either bivalirudin (n = 3) or heparin (n = 7). Aliquots from the heparin-contaminated RNA samples were treated with 0.3U of heparinase and miRNA expression was compared between heparin-contaminated, heparinase-treated, and bivalirudin-contaminated samples.

2.8. Statistical analysis

Statistical analysis was performed with SPSS software v22.0. Data normality was assessed using the Shapiro-Wilk test. Gaussian-distributed data were analysed using parametric tests (t test with Welch's correction; paired t test; one-way ANOVA) and non-Gaussian data using non-parametric tests (Wilcoxon matched-pairs signed rank test). Correlations between variables were analysed with the Spearman's correlation test. Data are presented as mean and standard error of the mean (SEM) or median and interquartile ranges (IQR) where appropriate and a $p < 0.05$ was considered statistically significant.

3. Results

3.1. Study cohort

Plasma samples were collected from a total of 73 STEMI patients undergoing PPCI. Optimal coronary perfusion (TIMI 3 flow) was obtained in 67 (93.1%) patients following PPCI. Clinical baseline characteristics of the study cohort are presented in **Table 1**.

3.2. Identification and selection of candidate endogenous miRNA controls

From the 179 quantified miRNAs, 60 presented Cq values < 35 across all 6 time points in STEMI patients and controls and were used for expression stability analysis by NormFinder and BestKeeper software (**Supplemental figures 3A and 3B**). The four most stable miRNAs identified by each algorithm were selected and an additional miRNA screening was performed in 6 STEMI patient samples to identify the four most stable miRNAs among this subgroup of eight miRNAs for further validation (miR-425-5p, miR-877-5p, miR-181a-5p, and miR-155-5p; **Supplemental figure 3C**).

3.3. miR-425-5p is a stably expressed endogenous miRNA in STEMI patients

To validate the previously identified candidate endogenous miRNA controls, miRNA quantification was performed in samples from 34 STEMI patients collected at 24h post-PPCI, hence not contaminated with heparin. Amongst the 4 candidate miRNAs, miR-425-5p presented the best stability scores in NormFinder and BestKeeper and shared the best stability values in geNorm as well as the lowest CV with miR-181a-5p (**Table 2**). In the entire STEMI cohort (n = 70), miR-425-5p expression was not influenced by age, sex, traditional cardiovascular risk factors, renal function, magnitude of myocardial damage (as reflected by cardiac troponin levels), or antiplatelet agents (glycoprotein IIb/IIIa inhibitors) administered during PPCI (**Table 1**). In addition, miR-425-5p Cq values strongly and negatively correlated with total RNA concentration in patient samples ($r = -0.744$, $p < 0.0001$) but did not correlate with platelet count ($r = 0.033$, $p = 0.786$) or 12h cardiac troponin levels ($r = -0.096$, $p = 0.429$) (**Supplemental figure 4**). Therefore, miR-425-5p was selected as an endogenous control for RT-qPCR normalisation.

3.4. Heparin concentrations in RNA and plasma samples from STEMI patients are comparable and can be reduced by heparinase treatment of RNA samples

Heparin was detected by ELISA in both plasma and plasma-derived RNA samples collected prior to myocardial reperfusion from the same STEMI patients (n = 10). Heparin concentration was similar between plasma [mean($\pm$ SEM) = 1,387($\pm$ 266)pg/mL] and RNA samples [1,113($\pm$ 167.4)pg/mL], p = 0.247 (**Figure 2A**), suggesting significant heparin resistance to the process of RNA extraction from plasma. Treatment of heparin-contaminated RNA samples with 0.25U, 0.5U, and 1U of heparinase resulted in approximate reduction of 7% ($\pm$ 0.2%, p = 0.337), 30% ($\pm$ 0.1%, p = 0.001), and 42% ($\pm$ 0.1%, p < 0.001) in heparin concentration, respectively (**Figure 2B**), indicating a dose-dependent inhibitory effect of heparinase on heparin levels in RNA samples.

3.5. Heparin inhibits the global miRNA mean, cel-miR-39, and miR-425-5p expression

In STEMI patients receiving heparin (n = 3), global miRNA mean Cq values remained significantly elevated by 4 cycles immediately after heparin administration until 30min post myocardial reperfusion in comparison to 24h post-reperfusion levels (**Figure 3A**). *In vitro* treatment of the same RNA samples from these STEMI patients with heparinase I prior to reverse transcription reduced the global miRNA mean by 4 cycles, bringing Cq values to similar levels of controls (**Figure 3B**).

Similarly, *in vitro* addition of heparin to RNA samples from non-heparinised STEMI patients resulted in significantly increased cel-miR-39 and miR-425-5p Cq values in a dose-dependent fashion (**Figures 3C and 3D**). In fact, a heparin dose of 2U completely inhibited the detection of such miRNAs by RT-qPCR. In contrast, *in vitro*

treatment of heparin-contaminated RNA samples from STEMI patients with doses as low as 0.25U and 0.5U of heparinase I was effective in reducing Cq values of both cel-miR-39 ($\Delta Cq = -2.7$, 95% CI: -2.1 to -3.3, $p < 0.0001$) and miR-425-5p ($\Delta Cq = -1.6$, 95% CI: -0.5 to -2.7, $p < 0.01$), respectively (**Figures 3C and 3D**).

Furthermore, cel-miR-39 expression in heparin-contaminated RNA samples from STEMI patients ($n = 35$) was significantly inhibited by 1.5 cycles in comparison to non-heparinised samples from healthy and stable CAD controls ($n = 12$), which were spiked-in with the same number of cel-miR-39 copies (**Figure 3E**). Finally, *in vivo* administration of heparin to STEMI patients ($n = 70$) inhibited miR-425-5p expression by 2.4 cycles in comparison to heparinase-treated samples from the same patients (**Figures 3F**).

3.6. Bivalirudin does not affect cel-miR-39 or miR-425-5p expression

Given heparin interference with all RT-qPCR normalisation approaches, the effect of bivalirudin, an anticoagulant that can be used alternatively during PPCI, on miR-425-5p and cel-miR-39 expression was also investigated. *In vitro* addition of bivalirudin to RNA samples from control stable CAD patients did not affect miR-425-5p or cel-miR-39 detection by RT-qPCR (**Figure 4A**). The effect of *in vivo* bivalirudin administration on miRNA expression in STEMI patients was assessed by comparing miR-425-5p expression between patients who received bivalirudin prior to PPCI and heparinase-treated RNA samples from STEMI patients across 13 different time points prior to and after PPCI. No statistically significant difference in miR-425-5p expression was observed between these two groups at any time point (**Figure 4B**).

3.7. Effect of RNA sample treatment with heparinase on cardiac-enriched miRNA expression

To test whether the inhibitory effect of heparin on RT-qPCR normalisation strategies had any impact on cardiac-enriched miRNA quantification, miR-1 and miR-133b were quantified in heparinase-treated (0.3U) and heparin-contaminated samples collected at 30min post-PPCI from the same patients (n = 70) and RT-qPCR data normalised to miR-425-5p. Median miR-1 and miR-133b levels were 3-fold and 1.5-fold higher in heparin-contaminated in comparison to heparinase-treated samples ($p < 0.0001$), respectively (**Figure 5A**). This overestimation of cardiac-enriched miRNA levels in heparin-contaminated samples could partially be a result of the direct inhibition of heparin on the normalisation control (miR-425-5p) Cq values. Nonetheless, the different magnitudes of change in miR-1 and miR-133b expression after heparinase treatment suggested that heparin could also directly affect cardiac-enriched miRNA detection in distinct degrees. Indeed, miR-133b expression was significantly reduced by *in vitro* treatment with low doses of heparin whereas treatment of heparin-contaminated RNA samples with heparinase was effective in improving miR-133b expression (**Figure 5B**). In contrast, higher, supra-physiological *in vitro* doses of heparin were required to significantly inhibit miR-1 expression and therefore heparinase treatment had no significant effect in restoring miR-1 Cq values in heparin-contaminated patient samples (**Figure 5C**).

In addition, correlation of miR-1 and miR-133b with 12h post-PPCI, high-sensitivity cardiac troponin T was compared between heparinase-treated and heparin-contaminated samples from the same STEMI patients. Treatment of RNA samples with heparinase improved the correlation between miR-1 and miR-133b levels at 30 minutes post-PPCI and 12h Troponin T (miR-1: $r = 0.602$, $p < 0.0001$ vs. $r = 0.510$, p

< 0.0001 ; miR-133b: $r = 0.540$, $p < 0.0001$ vs. $r = 0.520$, $p < 0.0001$) (**Figures 5D and 5E**). Correlations between these cardiac-enriched miRNA levels at 90min post-PPCI and troponin T were also evaluated in heparinase-treated samples ($n = 70$) and were slightly higher than those observed at 30min post-PPCI (miR-1: $r = 0.692$, $p < 0.0001$; miR-133b: $r = 0.647$, $p < 0.0001$). Expression of miR-1 and miR-133b were highly correlated, suggesting a common source for these miRNAs (**Supplemental table 3**).

4. Discussion

To the best of our knowledge, this is the first study to (i) identify and systematically validate a circulating miRNA as an endogenous control for RT-qPCR normalisation in STEMI patients; (ii) demonstrate that heparin administration simultaneously affects all currently proposed RT-qPCR normalisation strategies; (iii) show that bivalirudin does not affect the expression of exogenous or endogenous miRNA normalisation controls; (iv) validate the treatment of heparin-contaminated RNA samples with heparinase combined with RT-qPCR normalisation to miR-425-5p as a suitable approach for circulating miRNA quantification in a cohort of STEMI patients.

The paucity of standardized procedures for circulating miRNA quantification, especially regarding RT-qPCR data normalisation, might explain discrepancies or reproducibility issues amongst circulating miRNA studies (18). Some studies in patients with acute myocardial infarction have used endogenous miRNAs, such as U6 snRNA (19-22), miR-16 (23), and miR-17 (24, 25), as normalisation controls. However, these miRNAs are not stably detected in the blood (U6 snRNA) (26), are susceptible to haemolysis (miR-16) (27), or were deemed stable based only on no statistical difference in expression between a small cohort of STEMI patients and

controls (miR-17) (24). Selection of a reference miRNA solely on the basis of no statistically significant difference in expression between groups is not sufficient to establish that the miRNA is a stable reference and should involve more detailed *in silico* analysis (10). Thus, an unequivocally stable circulating endogenous miRNA control remained to be validated.

In this study, miR-425-5p was identified as a stably expressed miRNA using a robust statistical approach based on 3 established gene stability assessment software. In addition, miR-425-5p expression reflected RNA sample concentration, allowing this miRNA to correct for differences in the input RNA quantity amongst samples. This is highly valuable for circulating miRNA quantification considering that current miRNA reverse transcription protocols are based on same RNA input volume across different samples rather than same RNA concentration and that exogenous spike-in controls, although reflecting RNA extraction efficiency, are not able to correct for sources of variability such as input RNA quantity (10). Interestingly, miR-425-5p has also been validated as an endogenous control in patients with breast cancer (28). Circulating levels of miR-425-5p have otherwise been shown to be elevated in patients with colorectal (29) and cervical cancer (30) as well as in patients with traumatic brain injury (31), conditions that represented exclusion criteria for the present study. Data from miRNA tissue expression libraries (miRWalk 2.0, miRmine, miRGator 3.0) indicate high miR-425-5p expression in lymphoid cells, mammary glands, nasopharynx epithelium and mucosa, skin, brain, and testicular tissue (32-34). In the context of STEMI, the cellular or tissue sources of miR-425-5p remain to be elucidated in future studies. Our data suggest that miR-425-5p is not significantly expressed in or released from the myocardium in STEMI patients as there was no correlation between this miRNA plasmatic expression and circulating levels of

cardiac troponin T. In contrast, strong correlations were observed between known cardiac-enriched miRNAs (miR-1 and miR-133b) and troponin T. In addition, data from validated miRNA tissue expression libraries showed irrelevant expression of miR-425-5p in the heart (32-34). Platelets have been previously shown to release their miRNA content, encapsulated in microparticles, following activation and aggregation in STEMI patients (35). In addition, it has been recently postulated that accurate miRNA quantification in stored plasma samples relies on efficient removal of residual platelets prior to cryopreservation, as freezing of plasma samples resulted in release of platelet-derived microparticles containing miRNAs, including miR-425-5p, which expression strongly correlated with platelet count (36). However, in our study there was no correlation between miR-425-5p expression and baseline platelet count. Furthermore, miR-425-5p expression was not influenced by administration of antiplatelet aggregation medications (glycoprotein IIb/IIIa inhibitors) during PPCI in our cohort.

In STEMI patients undergoing PPCI, intravenous heparin administration represents a major obstacle for miRNA quantification, given its known interference with essential components of qPCR reactions, such as DNA polymerases and magnesium ions, resulting in qPCR inhibition (37, 38). Here, we demonstrate that heparin affects all currently proposed RT-qPCR normalisation strategies, including the global miRNA mean expression. This finding contradicts the study by Kaudewitz et al. (12), which reported that normalisation to the average Cq value of quantified miRNAs was able to overcome heparin-related cel-miR-39 inhibition and consequent overestimation of circulating miRNA levels in patients with acute coronary syndrome (ACS). In that study, however, miRNA mean expression was calculated based on the Cq values of only 14 miRNAs, some of which are known to be deregulated in patients with ACS.

In accordance with our findings, inhibition of cel-miR-39 detection within the initial hour of heparin administration has been previously reported in smaller cohorts of patients undergoing coronary angiography (11, 12).

On top of its general inhibition over PCR reactions, a selective inhibitory effect of heparin on endogenous circulating cardiovascular-related miRNAs detection by RT-qPCR has been reported by Boeckel et al. (11). In that study, miR-1, miR-92a, miR-126, miR-17, and miR-145 expression was not significantly altered after heparin administration to patients undergoing cardiac catheterisation (n = 11), whereas miR-133a, miR-34a, miR-378, and miR-499 detection was significantly reduced. No interference in miRNA detection was observed after addition of recombinant miR-92a and miR-126 to heparin-contaminated plasma samples as opposed to significant inhibition following addition of recombinant cel-miR-39 to the same samples, further suggesting an inhibitory effect of heparin towards specific miRNAs (11). Similarly, in our study, higher *in vitro* doses of heparin were required to inhibit miR-1 expression in non-heparinised STEMI samples when compared to miR-133b and miR-425-5p. This might explain why *in vitro* RNA treatment with heparinase was not effective in restoring miR-1 detection in heparin-contaminated samples from STEMI patients, in which heparin concentrations are lower than those necessary to inhibit miR-1 expression in our *in vitro* experiments. Consequently, following heparinase treatment of heparin-contaminated RNA samples from STEMI patients and PCR data normalisation to miR-425-5p, we observed different magnitudes of change in miR-1 and miR-133b expressions. A more pronounced reduction in miR-1 expression (3-fold) probably reflects the isolated effect of heparinase treatment in improving the endogenous normalisation control detection whereas the discreet reduction in miR-133b expression (1.5 fold) might result from a synergistic improving effect of

heparinase treatment on both the normaliser (miR-425-5p) and miR-133b detection. The mechanism behind this selective effect of heparin on miRNA detection is unknown. Future studies should investigate whether and how heparin interacts with the chemical structure of different miRNAs or plasmatic miRNA-binding proteins, such as Argonaute proteins (39).

Previous studies have also demonstrated that treatment of cellular and circulating RNA samples with heparinase could overcome heparin-induced RT-qPCR inhibition (40, 41). Whilst this manuscript was under preparation, Li S et al. (13) reported that heparinase treatment of RNA samples could abrogate heparin-induced impaired detection of cel-miR-39 in patients with CAD undergoing coronary intervention. Our data not only corroborate these findings but also show that treatment of heparin-contaminated RNA samples with heparinase improves the global miRNA mean and endogenous miRNA control (miR-425-5p) detection by RT-qPCR in STEMI patients post-PCI.

In this study, we also sought to explore whether bivalirudin had any effect on the expression of endogenous and exogenous miRNA normalisation controls. Bivalirudin is an anti-thrombotic medication that can be used for anticoagulation therapy during PCI in STEMI patients, especially in those with heparin-induced thrombocytopenia (42). *In vitro* and *in vivo* bivalirudin administration did not interfere with miR-425-5p or cel-miR-39 expression. This suggests that RNA samples obtained from patients treated with bivalirudin could be an alternative to circumvent heparin interference with RT-qPCR normalisation for clinical studies of circulating miRNAs in STEMI patients.

In addition, we observed that RNA treatment with heparinase improved the correlation of cardiac-enriched miRNAs post-reperfusion levels with high-sensitivity troponin T. Although elevated cardiac-enriched miRNA levels have been shown in STEMI patients, it remains controversial whether they correlate with the extent of cardiac injury (19, 43, 44). Data from the very few studies that performed serial cardiac-enriched miRNA quantification post-PPCI suggest that peak levels of these miRNAs occur in the initial 3-4h post-PPCI and correlated with markers of cardiomyocyte necrosis (20, 24, 45). Because this time window coincides with that of heparin's plasmatic half-life, our results allow future studies to overcome RT-qPCR inhibition and normalisation issues to carry out more detailed kinetics analysis in the initial hours post-PPCI and investigate whether cardiac-enriched miRNA levels after reperfusion reflect myocardial damage assessed by cardiac imaging.

This study presents some limitations. High throughput qPCR panels were used instead of microarray or RNA sequencing approaches for miRNA profiling and therefore could not identify novel miRNAs to be tested as stable endogenous controls. Furthermore, we did not evaluate whether miR-181a-5p, the second most stable endogenous miRNA, could produce similar results to miR-425-5p in terms of cardiac-enriched miRNA normalisation either separately or in association with miR-425-5p. Finally, we could not demonstrate a significant reduction in heparin concentration in RNA samples treated with 0.25U of heparinase by ELISA, whilst this dose was effective in restoring miRNA detection by RT-qPCR. We hypothesise that, due to RT-qPCR very high sensitivity, miRNA detection by RT-qPCR can be influenced by even small variations in heparin concentration in RNA samples, which ELISA might not be sensitive enough to detect.

In conclusion, this study addressed important methodological hurdles to accurate quantification of circulating miRNAs in STEMI patients. Our results have also implications for circulating miRNA studies in other cohorts to which anticoagulation therapy is administered, e.g. patients undergoing organ transplantation or heart surgery. In addition, we reinforce the evidence that levels of cardiac-enriched miRNAs early after myocardial reperfusion reflect the severity of cardiac injury. Finally, we suggest that the use of samples from bivalirudin-treated patients or *in vitro* treatment of heparin-contaminated samples with heparinase, associated with normalisation to miR-425-5p, are suitable strategies for miRNA quantification in STEMI patients, thus providing new tools to reduce variability and allow future detailed kinetics studies of circulating miRNAs in this population.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Figure legends

Figure 1. Study design. The study was carried out in three consecutive phases. In the screening phase (**A**), a total of 179 microRNAs were initially quantified in samples from 3 STEMI patients and 4 control samples using real time quantitative reverse transcription (RT-qPCR) panels to identify 8 candidate endogenous miRNA controls. In a second step of this phase, miRNA screening was performed in samples collected at 30min post-PCI from 6 STEMI patients and subsequently treated *in vitro* with heparinase to select the 4 most stable candidate endogenous miRNAs and to assess the effect of heparin and heparinase administration on the global miRNA mean expression (**B**), the 4 candidate miRNAs previously identified were quantified by TaqMan RT-qPCR in 34 STEMI samples collected 24h post-PCI (heparin-free) and miRNA stability was determined. In addition, cel-miR-39 and the validated endogenous miRNA control were quantified in a total of 70 STEMI samples collected at 30min post-PCI (cel-miR-39, n = 35; endogenous miRNA control, n = 70) to evaluate the effect of *in vivo* administration of heparin on these miRNA controls expression. To further assess the *in vivo* effect of bivalirudin on the exogenous spike-in and endogenous miRNA controls, these miRNAs were quantified in samples collected from STEMI patients (n = 3) at 13 time points prior to and following PCI. Finally, in the application phase (**C**), heparin-contaminated RNA samples from the same 70 STEMI patients (30min post-PCI) were treated *in vitro* with heparinase and cardiac-enriched miRNAs (miR-1 and miR-133b) were quantified to assess the impact of heparin contamination and heparinase treatment on the levels of such miRNAs. Furthermore, correlation of cardiac-enriched miRNA levels with 12h post-PPCI Troponin T was also analysed.

Figure 2. *In vitro* treatment with heparinase decreases heparin concentration in RNA samples from STEMI patients. Heparin was quantified in plasma and plasma-derived RNA samples collected prior to myocardial reperfusion from STEMI patients by ELISA. (**A**) Presence of heparin was confirmed in both plasma and RNA samples. Heparin concentration was similar between plasma [mean($\pm$ SEM) = 1,387($\pm$ 266)pg/mL] and RNA samples [1,113($\pm$ 167.4)pg/mL], $p = 0.247$; $n = 10$; ns, non-significant; paired t test. (**B**) Serial doses of heparinase I (0.25U, 0.5U, and 1U) were added to heparin contaminated samples from STEMI patients ($n = 3$), incubated for 1 hour at room temperature, and heparin concentration was subsequently assessed by ELISA. Addition of 0.5U and 1U were effective in reducing heparin concentration by approximately 30% and 42%, respectively, in relation to heparin-contaminated samples not treated with heparinase; $**p=0.001$ $***p<0.001$ vs. control samples, one-way ANOVA and Dunnett's multiple comparison test.

Figure 3. Heparin inhibitory effect on the global miRNA mean, cel-miR-39, and miR-425-5p expression. (A) STEMI patients treated with heparin displayed significantly higher global miRNA mean Cq across the initial 30min following myocardial reperfusion in comparison to 24h values, when heparin was not present in the circulation; n = 3; ns, non-significant, ****p<0.0001 vs 24h; Repeated-measures one-way ANOVA and Dunnett's multiple comparison test; (B) To confirm *in vitro* whether heparin inhibits the global miRNA mean expression, heparin-contaminated RNA samples were treated with 0.3U of heparinase prior to reverse transcription and the difference in the global miRNA mean between these samples and non-heparinised, stable CAD controls (Δ Cq) was calculated. Heparin-contaminated samples not treated with heparinase presented global miRNA mean 4 cycles higher than controls whereas samples from the same patients that were treated with heparinase had similar miRNA mean Cq to controls; n = 3; ns, non-significant, ****p<0.0001 vs stable CAD controls; one-way ANOVA and Dunnett's multiple comparison test; (C, D) Effect of *in vitro* addition of serial doses of heparin or heparinase to RNA samples on cel-miR-39 and miR-425-5p expression. Seven different doses of heparin were added to RNA samples from STEMI patients not treated with heparin and changes on cel-miR-39 and miR-425-5p Cq were compared to the control, heparin-free samples (Δ Cq). Heparin significantly inhibited both cel-miR-39 and miR-425-5p expression, whereas 2U completely inhibited miRNA detection by RT-qPCR. In contrast, treatment of heparin-contaminated samples from STEMI patients with 5 different doses of heparinase reduced Cq values, n = 3; ns, non-significant, *p<0.05, **p<0.01, ****p<0.0001; one-way ANOVA and Dunnett's multiple comparison test; (E, F) *In vivo* effect of heparin administration on cel-miR-39 and miR-425-5p expression. Cel-miR-39 expression was significantly inhibited in heparin-contaminated STEMI samples (n = 35) in comparison to non-heparinised samples from healthy and stable CAD controls (n = 12), which were spiked-in with the same number of cel-miR-39 copies ***p<0.001, Mann-Whitney U test. Similarly, miR-425-5p expression was inhibited by 2.4 cycles in heparin-contaminated versus heparinase-treated RNA samples from the same patients, n = 70; ****p<0.0001, Wilcoxon matched-pairs signed rank test.

Figure 4. Bivalirudin does not affect miR-425-5p or cel-miR-39 expression. (A) *In vitro* addition of bivalirudin to RNA samples from patients with stable coronary artery disease did not result in changes on miR-425-5p or cel-miR-39 Cq values, n = 3; ns, non-significant, one-way ANOVA and Dunnett's multiple comparison test; (B) To evaluate the effect of bivalirudin on miR-425-5p *in vivo*, samples were obtained at 13 different time points from STEMI patients receiving either bivalirudin (n = 3) or heparin (n = 7). Aliquots from the heparin-contaminated RNA samples were treated with 0.3U of heparinase and miR-425-5p expression was compared between heparin-contaminated, heparinase-treated, and bivalirudin-contaminated samples. Significantly reduced Cq values were observed in heparinase-treated samples in comparison to heparin-contaminated samples from the same patients up to 30min post-PCI; *p<0.05, Wilcoxon matched-pairs signed rank test. No statistically significant differences were observed in miR-425-5p expression between

heparinase-treated and bivalirudin-contaminated samples; ns, non-significant, Mann-Whitney U test.

Figure 5. Effect of RNA sample treatment with heparinase on cardiac-enriched miRNA expression. (A) To assess whether RNA treatment with heparinase could affect cardiac-enriched miRNA quantification, miR-1 and miR-133b were quantified in heparinase-treated (0.3U) and heparin-contaminated samples collected at 30min post-PPCI from the same patients and data normalised to miR-425-5p. Expression of miR-1 and miR-133b in heparinase-treated samples were 3-fold and 1.5-fold lower than that of heparin-contaminated samples; $n = 70$, **** $p < 0.0001$; Wilcoxon matched-pairs signed rank test; **(B, C)** Effect of *in vitro* addition of serial doses of heparin or heparinase to RNA samples on miR-133b and miR-1 expression. Expression of miR-133b was affected by almost all tested doses of heparin and heparinase. In contrast, miR-1 Cq values were only affected by higher, supra-physiological doses of heparin and not significantly affected by heparinase in heparin-contaminated patient samples, $n = 3$; ns, non-significant, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ vs controls; one-way ANOVA and Dunnett's multiple comparison test; **(D, E)** Effect of heparinase treatment on the correlation of cardiac-enriched miRNAs, miR-1 and miR-133b, with 12h post-PPCI troponin T, $n = 70$.

Extra table

What is known in this topic
<ul style="list-style-type: none">• Lack of consensus regarding RT-qPCR normalisation for miRNA quantification in STEMI patients contributes to irreproducibility amongst studies and impairs translation of miRNAs to clinical practice• There is no validated endogenous miRNA normalisation control in STEMI patients• Heparin inhibits the detection of the commonly used exogenous miRNA normalisation control cel-miR-39
What this paper adds
<ul style="list-style-type: none">• We identified and validated miR-425-5p as a stable endogenous miRNA control in STEMI patients• Besides its effect on cel-miR-39, heparin also interferes with the other two currently proposed RT-qPCR normalisation strategies for miRNA quantification (i.e. global miRNA mean and miRNA endogenous control)• RNA samples from bivalirudin-treated STEMI patients or <i>in vitro</i> treatment of heparin-contaminated samples with heparinase can be used to circumvent heparin-related issues in RT-qPCR inhibition and normalisation

Table 1. Clinical characteristics of the study population

Variable	Entire cohort	Endogenous miR control Cq values*			p value
		1 st tertile	2 nd tertile	3 rd tertile	
Number (male)	<u>73 (61)</u>	<u>24 (20)</u>	<u>25 (20)</u>	<u>24 (21)</u>	<u>0.898</u>
Age [years, median (IQR)]	<u>63 (55 – 71)</u>	<u>65 (56 – 71)</u>	<u>65 (59 – 70)</u>	<u>61 (52 – 71)</u>	<u>0.396</u>
Risk factors, n (%)					
Smoking status					
Never smoked	<u>28 (38.9)</u>	<u>10 (41.7)</u>	<u>7 (29.2)</u>	<u>11 (45.8)</u>	<u>0.468</u>
Ex-smoker	<u>24 (33.3)</u>	<u>9 (37.5)</u>	<u>11 (45.8)</u>	<u>4 (16.7)</u>	<u>0.087</u>
Current smoker	<u>20 (27.8)</u>	<u>5 (20.8)</u>	<u>6 (25)</u>	<u>9 (37.5)</u>	<u>0.407</u>
Hypertension	<u>17 (23.6)</u>	<u>4 (16.7)</u>	<u>7 (29.2)</u>	<u>6 (25)</u>	<u>0.583</u>
Diabetes mellitus	<u>6 (8.3)</u>	<u>3 (12.5)</u>	<u>3 (12.5)</u>	<u>0 (0)</u>	<u>0.195</u>
Hypercholesterolaemia	<u>7 (9.7)</u>	<u>3 (12.5)</u>	<u>2 (8.3)</u>	<u>2 (8.3)</u>	<u>0.854</u>
Obesity	<u>25 (34.7)</u>	<u>8 (33.3)</u>	<u>13 (56.5)</u>	<u>4 (16.7)</u>	<u>0.230</u>
Malignancies	0 (0)	0 (0)	0 (0)	0 (0)	-
Laboratory tests [median (IQR)]					
Admission eGFR, mL/min	<u>83 (73 – 97)</u>	<u>82 (68 – 92)</u>	<u>83 (75 – 112)</u>	<u>83 (69 – 93)</u>	<u>0.441</u>
Admission Troponin T, ng/L	<u>49 (27 – 98)</u>	<u>33 (27 – 72)</u>	<u>61 (28 – 184)</u>	<u>46 (25 – 141)</u>	<u>0.346</u>
Peak Troponin T, ng/L	<u>3255 (1109 – 5658)</u>	<u>3422 (1712 – 6441)</u>	<u>2262 (745 – 6367)</u>	<u>3119 (1179 – 5152)</u>	<u>0.533</u>
STEMI characteristics					
Onset to reperfusion (min)	<u>157 (110 – 245)</u>	<u>144 (102 – 216)</u>	<u>225 (124 – 295)</u>	<u>147 (112 – 248)</u>	<u>0.156</u>
Culprit vessel, n (%)					
LAD	<u>22 (30.6)</u>	<u>6 (25)</u>	<u>10 (41.7)</u>	<u>6 (25)</u>	<u>0.351</u>
LCx	<u>13 (18)</u>	<u>5 (20.8)</u>	<u>3 (12.5)</u>	<u>5 (20.8)</u>	<u>0.687</u>
RCA	<u>37 (51.4)</u>	<u>13 (54.2)</u>	<u>11 (45.8)</u>	<u>13 (54.2)</u>	<u>0.801</u>
Localization, n (%)					
Anterior	<u>22 (30.6)</u>	<u>6 (25)</u>	<u>10 (41.7)</u>	<u>6 (25)</u>	<u>0.351</u>
Non-anterior	<u>50 (69.4)</u>	<u>18 (75)</u>	<u>14 (58.3)</u>	<u>18 (75)</u>	<u>0.351</u>
TIMI flow pre PPCI, n (%)					
0	<u>60 (83.3)</u>	<u>21 (87.5)</u>	<u>19 (79.2)</u>	<u>20 (83.3)</u>	<u>0.741</u>
1	<u>10 (13.9)</u>	<u>3 (12.5)</u>	<u>4 (16.7)</u>	<u>3 (12.5)</u>	<u>0.890</u>
2	<u>1 (1.4)</u>	<u>0 (0)</u>	<u>0 (0)</u>	<u>1 (4.2)</u>	<u>0.363</u>
3	<u>1 (1.4)</u>	<u>0 (0)</u>	<u>1 (4.2)</u>	<u>0 (0)</u>	<u>0.363</u>
TIMI flow post PPCI, n (%)					
0	<u>1 (1.4)</u>	<u>0 (0)</u>	<u>1 (4.2)</u>	<u>0 (0)</u>	<u>0.363</u>
1	<u>3 (4.2)</u>	<u>0 (0)</u>	<u>1 (4.2)</u>	<u>2 (8.3)</u>	<u>0.352</u>
2	<u>1 (1.4)</u>	<u>0 (0)</u>	<u>0 (0)</u>	<u>1 (4.2)</u>	<u>0.363</u>
3	<u>67 (93.1)</u>	<u>24 (100)</u>	<u>22 (91.7)</u>	<u>21 (87.5)</u>	<u>0.222</u>
Medication during PPCI					
Heparin, n (%)	<u>70 (95.8)</u>	<u>24 (100)</u>	<u>24 (96)</u>	<u>22 (91.7)</u>	<u>0.328</u>
Bivalirudin, n (%)	<u>3 (4.2)</u>	<u>0 (0)</u>	<u>1 (4)</u>	<u>2 (8.3)</u>	<u>0.328</u>
Glycoprotein IIb/IIIa inhibitors, n (%)	<u>48 (66.7)</u>	<u>15 (62.5)</u>	<u>18 (75)</u>	<u>15 (62.5)</u>	<u>0.570</u>

eGFR, estimated glomerular filtration rate; LAD, left anterior descending; LCx, left circumflex; PPCI, primary percutaneous coronary intervention; RCA, right coronary artery; TIMI, thrombolysis in myocardial infarction; * endogenous miRNA control Cq values at 30 minutes post myocardial reperfusion (expression quantified in heparinase-treated RNA samples, except in samples from bivalirudin-treated patients)

Table 2. Candidate endogenous miRNA controls expression stability

microRNA	geNorm M score	Normfinder S score	Bestkeeper Std dev ($\pm$CP)	CV (%)
miR-425-5p	1.13	1.85	1.47	7
miR-181a-5p	1.13	2.09	1.51	7
miR-155-5p	2.62	2.04	4.03	14
miR-877-5p	2.72	2.18	3.97	13

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

SUPPLEMENTS

1. Supplemental methods

1.1. RNA extraction

RNA was isolated from 200 μ L of plasma samples, which had been cryopreserved at -80°C for 6 months to 1 year prior to analysis (Freeze/thaw cycles = 2 to all samples). The miRNeasy serum/plasma kit (Qiagen, Germany) was used for RNA extraction, following the manufacturer's instructions. Fixed volumes of each of the kit's reagents used throughout the entire study were as follows:

Plasma sample	200 μ L
Qiazol reagent	1,000 μ L
Cel-miR-39 spike-in	3.5 μ L
Chloroform	200 μ L
Aqueous phase	500 μ L
100% Ethanol	750 μ L
RWT buffer	700 μ L
RPE buffer	500 μ L
80% Ethanol	500 μ L
RNAse free water	
(for final RNA elution).....	60 μ L

Following RNA extraction, quantification of total RNA in each sample was performed using RNA 6000 Pico Kit (Agilent technologies, Germany) by a 2100 Bioanalyzer instrument (**Supplemental table 1**). RNA integrity was assessed by the RNA integrity number (RIN). Quality assessment in RNA samples obtained from plasma is challenging because of the very low yield of RNA in these samples and therefore RIN must be interpreted with caution, as recommended by the MIQE guidelines.

1.2. Heparin ELISA

Heparin was quantified in plasma and RNA samples using a competitive enzyme-linked immunosorbent assay (ELISA) kit (BlueGene, China), containing wells pre-coated with monoclonal anti-porcine heparin antibody. Plasma (100µL) and RNA (10µL; diluted in 90µL of PBS to a final volume of 100µL) samples were incubated with a heparin-horseradish peroxidase (HRP) conjugate in the pre-coated wells at 37°C for 1 hour. PBS only (100µL) was added to blank control wells and appropriate standards (100µL) containing six serial concentrations of heparin (range: 0pg/mL – 2,500pg/mL) were included in duplicates to generate a standard curve for heparin concentration calculation in STEMI samples. After the incubation period, the sample-conjugate solution was removed and wells were washed 6 times with 200µL of washing buffer. A substrate for HRP (100µL) was then added to each well and incubated at 37°C for 15 minutes, including the blank wells. Finally, a stop solution was added to the wells and absorbance was immediately measured at 450nm wavelength by spectrophotometry (Varioskan lux, Thermo Fisher Scientific, USA). A standard curve (below) was generated using a four-parameter logistic (4-PL) curve-fit. Heparin concentration in the samples was calculated based on the standard curve and dilution factor (RNA samples) by the SkanIt software (Thermo Fisher Scientific, USA).

$$y = 1.73465 + ((0.135615 - 1.73465) / (1 + (x/858.255)^{-1.1809}))$$

$$R^2 = 0.967$$

To test the effect of RNA sample treatment with heparinase on heparin concentration, 10µL of heparin-contaminated RNA samples from the same patients (n = 3) were incubated with different doses of heparinase (0U, 0.25U, 0.5U, and 1U) at room temperature for 1 hour prior to ELISA.

1.3. Reverse transcription

In the screening phase, RNA was reverse transcribed using the Universal cDNA synthesis kit II (Exiqon, Denmark), by mixing RNA samples with kit components followed by incubation in thermocycler according to the manufacturer's protocol, as indicated below:

RT reaction components	Volume (µL/per reaction)
RNA sample	4
5X Reaction buffer	4
Enzyme mix	2
UniSp6 spike-in	1
Nuclease free water	9
Total reaction volume	20

Thermocycler settings for RT reaction

Incubation 42°C..... 60min
Heat/Inactivation 95°C..... 5min

In the validation and application phases, cDNA was synthesized using the TaqMan[®] microRNA reverse transcription kit (Applied Biosystems, USA) and stem-loop TaqMan miRNA primers (Applied Biosystems, USA), as follows:

RT reaction components	Volume (μ L/per reaction)
RNA sample	5
dNTPs	0.15
Reverse transcriptase	1
10X RT buffer	1.5
RNAse inhibitor	0.19
Nuclease free water	4.16
TaqMan 5X miRNA assay	3
Total reaction volume	15

Thermocycler settings for RT reaction

Incubation 16°C..... 30min

Incubation 42°C..... 30min

Heat/Inactivation 85°C..... 5min

For each assay, a control containing all reagents apart from reverse transcriptase was included to assess the presence of DNA contamination.

1.4. Real-time qPCR

Only fresh cDNA samples were used for real-time qPCR amplification. In the screening phase, a panel of 179 miRNAs were quantified using serum/plasma focused miRNA PCR panels (Exiqon, Denmark) containing different primers for the target miRNAs in each well. Volumes of reaction components and qPCR conditions are described below:

qPCR reaction components	Volume (μL /per plate)
cDNA sample	10
Exilent SYBR Green master mix	500
Nuclease free water	490
Total volume per plate	1000
Total volume per well	10

7500AB instrument settings for qPCR reaction

Polymerase activation/denaturation..... 95°C..... 10min
Denaturation (40 cycles) 95°C..... 10sec
Annealing/extension (40 cycles) 60°C..... 60sec
Melting curve analysis

In the validation and application phases, TaqMan small RNA assays and the SensiFAST probe Hi-ROX master mix (Bioline, UK) were used for qPCR in triplicates:

qPCR reaction components	Volume (μL /per triplicates)
cDNA sample	4.8
TaqMan small RNA assay (20X)	3.6
SensiFAST probe Hi-ROX master mix	36
Nuclease free water	27.6
Total volume	72
Total volume per well	20

7500AB instrument settings for qPCR reaction

Polymerase activation/denaturation..... 95°C..... 10min

Denaturation (40 cycles) 95°C..... 15sec

Annealing/extension (40 cycles) 60°C..... 60sec

For each of the 9 TaqMan small RNA assays used in this study, calibration curves based on cDNA serial dilutions (Dilution factor = 4) were produced for the assessment of primer efficiency and qPCR linear dynamic range. Primer efficiency (E%) was calculated as a function of the calibration curves slopes; $E = 10^{(-1/\text{slope})}$. All assays presented E% between 95% and 105% as well as coefficient of determination (R^2) > 0.98, indicating optimal qPCR amplification (**Supplemental figure 2**). In addition, intra-assay variability across biological replicates was also determined for all miRNA assays (**Supplemental table 2**). Finally, two types of non-template controls were included for each assay: (i) no reverse transcriptase controls, as mentioned above, to evaluate genomic DNA contamination; (ii) controls in which cDNA template was replaced by nuclease free water, to assess background noise. These controls displayed undetected Cq values in all plates.

The comparative Cq ($\Delta\Delta Cq$) method was used to determine Cq values and real-time qPCR data was analyzed using the 7500 software v2.0.5. Fold changes were calculated using the $2^{-\Delta\Delta Cq}$ method.

1.5. *In vitro* experiments with heparin, heparinase, and bivalirudin

To evaluate whether the *in vitro* addition of heparin to heparin-free RNA samples from STEMI patients could affect miRNA detection, serial doses of heparin sodium (1,000 I.U/mL; Sigma-Aldrich) were added to 5µL aliquots from the same RNA

patient samples (n = 3) and the reverse transcription reaction components prior to reverse transcription according to the following scheme:

Sample	Heparin concentration (U)
Control (Heparin-free)	0
1	2
2	1
3	0.5
4	0.25
5	0.1
6	0.05
7	0.005

RT reaction components	Volume (μL/per reaction)
RNA sample	5
Heparin	1
dNTPs	0.15
Reverse transcriptase	1
10X RT buffer	1.5
RNAse inhibitor	0.19
Nuclease free water	3.16
TaqMan 5X miRNA assay	3
Total reaction volume	15

To test whether RNA sample treatment with heparinase could abrogate heparin effects and identify an appropriate dose, aliquots from the same heparin-

contaminated RNA samples from STEMI patients (n = 3) were incubated with serial doses of heparinase I from *Flavobacterium heparinum* (Sigma-Aldrich), 10x reverse transcription buffer, and RNase inhibitor for 1 hour at room temperature, as previously described by Johnson ML et al. (1) and Li S et al.(2):

Sample	Heparinase concentration (U)
Control (heparin-contaminated)	0
1	1
2	0.25
3	0.5
4	0.1

RT reaction components (Part 1: incubation with heparinase)	Volume (µL/per reaction)
RNA sample	5
Heparinase	1
10X RT buffer	1.5
RNase inhibitor	0.19

After incubation, the remaining components of the reverse transcription reaction were added in the same volumes as described above for the heparin spike-in experiment and reverse transcription was performed:

RT reaction components (Part 2)	Volume (µL/per reaction)
dNTPs	0.15
Reverse transcriptase	1
Nuclease free water	3.16
TaqMan 5X miRNA assay	3

For the bivalirudin spike-in experiment, 1 μ L of bivalirudin (Sigma-Aldrich) at the concentrations of 0.1 and 1 μ g/ μ L were added to aliquots of the same RNA samples from stable coronary artery disease patients (n = 3) prior to reverse transcription as follows:

RT reaction components	Volume (μ L/per reaction)
RNA sample	5
Heparin	1
dNTPs	0.15
Reverse transcriptase	1
10X RT buffer	1.5
RNAse inhibitor	0.19
Nuclease free water	3.16
TaqMan 5X miRNA assay	3
Total reaction volume	15

Supplemental figure 1. MicroRNA screening data quality control. Three synthetic microRNA spike-ins were equally added to all samples included in the screening phase to assess the efficiency of each experimental step: RNA extraction (cel-miR-39), cDNA synthesis (UniSp6), and qPCR (UniSp3). Expression of cel-miR-39 was significantly lower up to 5 minutes post-reperfusion, with a trend of higher Cq values up to 30 minutes, in comparison to non-heparinised stable coronary artery disease (CAD) control samples (A), most likely reflecting reduced cel-miR-39 qPCR detection as a result of heparin inhibition rather than true differences in RNA extraction efficiency between samples. Data expressed as median and interquartile range; *p<0.05; Kruskal-Wallis test with Dunn's correction for multiple comparisons. (B) Expression of UniSp6 was also significantly decreased up to 5 minutes post-reperfusion in STEMI patients receiving heparin in comparison to controls. Data expressed as median and interquartile range; *p<0.05; Kruskal-Wallis test with Dunn's correction for multiple comparisons. (C) UniSp3 detection was very stable amongst all samples, indicating similar qPCR efficiency. UniSp3 Cq values were used as a normalizer for inter-plate calibration to minimize inter-run variability.

Supplemental figure 2. Taqman small RNA assay calibration curves. Serial cDNA template dilutions (dilution factor = 4) were performed and calibration curves were plotted. The coefficient of determination (R^2) and slope for each curve are displayed. Primer efficiency (E%) was calculated as a function of the slope, $E = 10^{(-1/\text{slope})}$. Quantification cycle (Cq) values are displayed as the average of 3 technical replicates.

2. Supplemental Results

Supplemental Figure 3. Selection of candidate endogenous miRNA controls following screening. After miRNA screening in 3 STEMI patients and 4 controls, 60 miRNAs presented Cq values < 35 across all time points and had their stability determined by NormFinder and BestKeeper. The four most stable miRNAs below the M score threshold of 0.5 identified by NormFinder **(A)** and the four most stable miRNAs with standard deviation < 1 in BestKeeper **(B)** were selected for further validation. Note that cel-miR-39 is amongst the least stable miRNAs in both algorithms. **(C)** Following screening of 179 circulating miRNAs in 6 samples collected from STEMI patients 30min post percutaneous coronary intervention (PCI), which were treated in vitro with 0.3U of heparinase, and 4 controls with stable coronary artery disease (CAD), the 4 most stably expressed endogenous miRNAs (miR-425-5p, miR-877-5p, miR-181a-5p, miR-155-5p - highlighted) were identified by NormFinder. The lower the stability (S) value in NormFinder the more stable the reference miRNA. These miRNAs were selected for further validation by TaqMan RT-qPCR assays in a larger cohort of STEMI patients.

Supplemental Figure 4. Correlation of miR-425-5p with sample RNA concentration and platelet count. (A) miR-425-5p expression strongly and negatively correlated with total RNA concentration in STEMI patient samples, $n = 70$. (B) miR-425-5p expression at 30min post-PPCI did not correlate with admission platelet count, $n = 70$. (C) miR-425-5p expression at 30min post-PPCI did not correlate with peak cardiac troponin T, $n = 70$.

Supplemental table 1. Total RNA concentration and integrity assessment

Sample	Agilent 2100 Bioanalyzer		Sample	Agilent 2100 Bioanalyzer	
	RNA conc. (pg/ μ L)	RIN		RNA conc. (pg/ μ L)	RIN
ID001 30min	1062.9	2.7	ID146 90min	1020.7	2.8
ID001 90min	604.3	2.5	ID147 30min	199.7	2.5
ID002 30min	571.4	3	ID147 90min	350.6	2.7
ID002 90min	1065.9	3.2	ID148 30min	357.2	2.6
ID003 30min	3217.5	3.9	ID148 90min	743	2.8
ID003 90min	3660.7	4.5	ID149 30min	153.1	2.5
ID004 30min	2249.2	3.3	ID149 90min	195.8	2.5
ID004 90min	3719.9	3.4	ID150 30min	758.4	2.6
ID005 30min	362.5	2.5	ID150 90min	616.6	2.7
ID005 90min	611.2	2.7	ID152 30min	523.9	3
ID006 30min	341.3	2.6	ID152 90min	1666	2.6
ID006 90min	552.3	3	ID153 30min	710.9	2.7
ID007 30min	280	2.4	ID153 90min	346.5	2.7
ID007 90min	389.5	3	ID154 30min	173	2.8
ID008 30min	309.2	2.3	ID154 90min	657	2.9
ID008 90min	309.7	2.7	ID206 30min	2230.6	3.5
ID009 30min	395.9	2.7	ID206 90min	2898.3	4.6
ID009 90min	513.5	2.9	ID207 30min	213	2.5
ID010 30min	100.6	2.5	ID207 90min	407.1	2.7
ID010 90min	320.2	2.5	ID501 30min	338.2	3.4
ID012 30min	900.7	3.3	ID501 90min	292	3
ID012 90min	613.9	3.1	ID570 30min	581.8	2.6
ID069 30min	203	2.8	ID570 90min	453.6	2.8
ID069 90min	1113.5	3	ID571 30min	569.2	2.5
ID138 30min	428	2.6	ID571 90min	392.7	2.5
ID138 90min	342	2.5	ID639 30min	364.9	2.6
ID141 30min	345.9	2.6	ID639 90min	554.4	2.6
ID141 90min	561.9	2.6	ID640 30min	339.6	2.5
ID142 30min	260	2.5	ID640 90min	368.9	2.6
ID142 90min	387	2.6	ID707 30min	1356.5	2.8
ID144 30min	519.9	2.6	ID707 90min	410.6	2.7
ID144 90min	264.1	2.5	ID708 30min	514.2	2.9
ID145 30min	1545.1	3.1	ID708 90min	413.7	2.9
ID145 90min	1797.5	3.3	ID709 30min	201	2.2
ID146 30min	624.2	2.6	ID709 90min	556	2.9

RIN, RNA integrity number

Supplemental table 2. MicroRNA intra-assay variability

microRNA	CV (%)
cel-miR-39	0.86
miR-425-5p	0.99
miR-155-5p	0.43
miR-181a-5p	1.47
miR-877-5p	3.97
miR-1	4.14
miR-133b	2.53

CV, coefficient of variability

Supplemental table 4. Correlation between miR-1 and miR-133b*

Time point post-PPCI	Spearman's <i>r</i>	<i>P</i> -value
30 min	0.934	< 0.0001
90min	0.949	< 0.0001

* Correlation based on miRNA fold-change to controls

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